Adenovirus Vectors in Gene Therapy

Advanced article

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Article Contents

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Adenovirus vectors are widely used for delivery of foreign deoxyribonucleic acid to mammalian cells. They are important tools in research and for use in gene therapy and vaccines.

Introduction

Adenoviruses (Ads) are used extensively to deliver genes into mammalian cells for a variety of different purposes, chief among them for research where high-level expression of transgene products in cultured cells is desired, for use as recombinant viral vaccines and for gene therapy. These viruses are particularly well suited for many applications because they are stable and grow to high titres, they are easy to manipulate and to purify and they can transduce many cell types from numerous mammalian species including both dividing and nondividing cells *in vitro* and *in vivo*.

Adenovirus Biology

The adenovirus family has numerous members distributed throughout the animal kingdom. Over 50 different serotypes have been isolated from humans alone but most research, including development of vectors, has focussed on just a few common serotypes, particularly human Ads 2 and 5. The virion is a nonenveloped icosahedral particle of approximately 100 nanometers (nm) in diameter, comprising a protein capsid surrounding core proteins and a linear double-stranded deoxyribonucleic acid (DNA) genome of approximately 30–40 kilobases (kb) (**Figure 1**). DNA replication and virion assembly take place in the nucleus of infected cells. The production of very large amounts of viral products results in cell death and release of several thousands of infectious viruses per cell at the end of the replication cycle. **See also:** Adenoviruses

Viral genome and viral life cycle

Figure 1 shows a simplified map of the Ad5 genome. The replication cycle of the virus can be divided into two phases: early, corresponding to events occurring before the onset of viral DNA replication; and late, corresponding to the period after initiation of DNA replication. During the early phase, four noncontiguous regions of the genome are expressed: early region 1 (E1), which comprises E1a and E1b, and E2, E3 and E4. After the onset of DNA replication, the major late promoter (MLP) located at 16 map units (mu) drives much of the viral transcription.

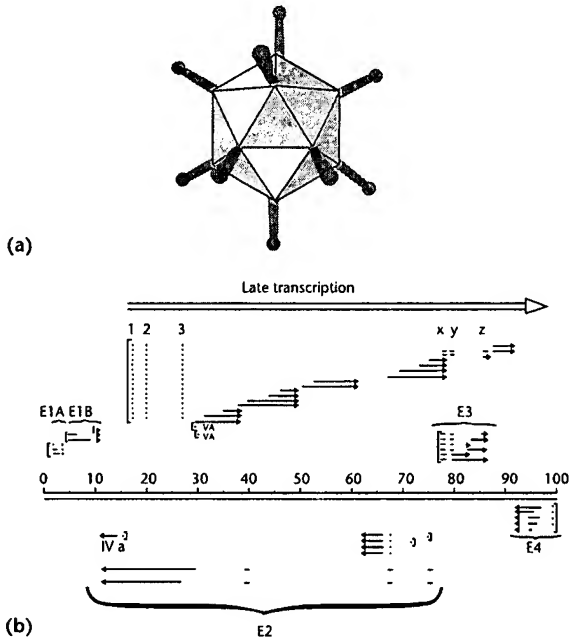

Figure 1 (a) Representation of the adenovirus virion. The Ad virion is an icosahedron with protrusions, called fibre, attached to penton base at each of the 12 vertices. The capsid protein that forms the major component of the 20 facets is called hexon. A dozen or so additional proteins make up the capsid and core of the virion. Approximately 15% of the molecular mass of the particle comprises DNA packaged as a linear double-stranded molecule. (b) Organization of the viral genome (100 map units (mu) = 36 kb). Promoters are indicated by square brackets. Transcription from the major late promoter at 16 mu generates a single long transcript that is spliced into late mRNAs as indicated. 1, 2, 3 and x, y, z represent leader RNAs attached to various late messages. Virus-associated (VA) RNAs are RNA polymerase III transcripts initiating around 29 mu. The mRNA for protein IVa2 is synthesized at intermediate times from a promoter at 16 mu.

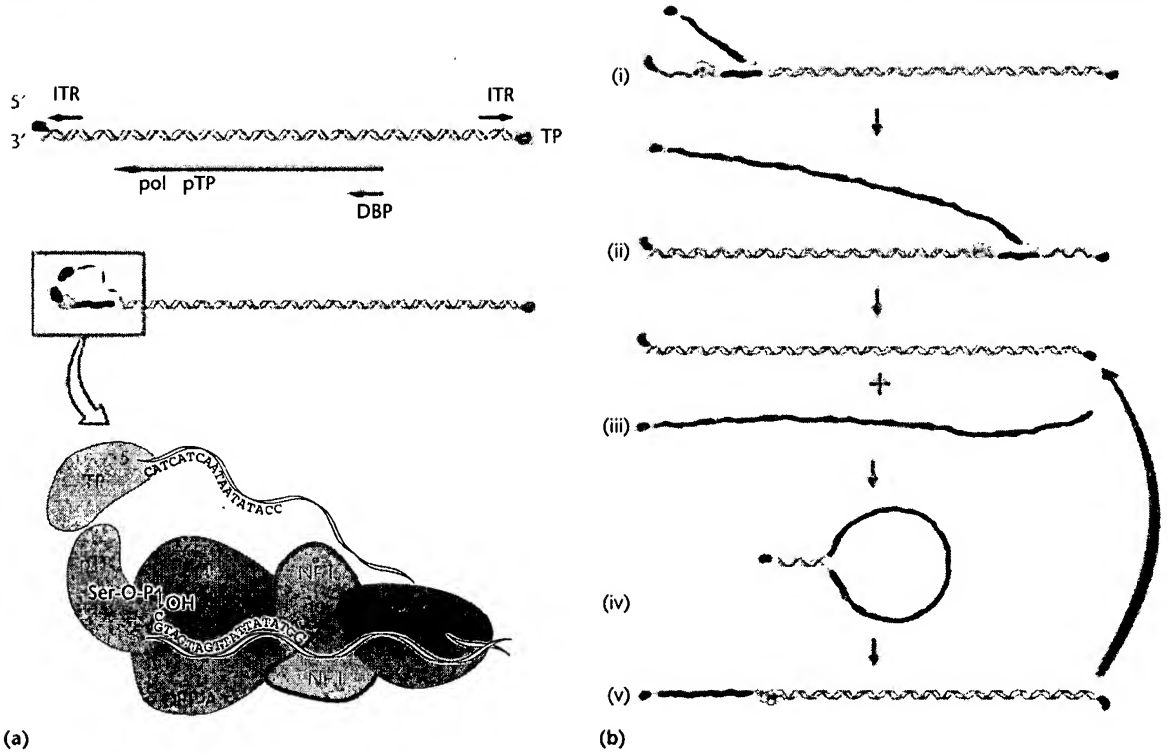

Figure 2 Adenovirus DNA replication. (a) Initiation of Ad DNA replication is protein primed and can occur at either end of the viral DNA. Viral DNA replication requires cellular proteins called ORP-A, NFI and NFIII (or Oct-1) in addition to the viral E2B region-coded DNA polymerase (pol) and preterminal protein (pTP). A serine residue in pTP becomes covalently linked to a deoxycytidine monophosphate (dCMP) residue in a reaction catalysed by the virus-coded pol. The 3′ hydroxyl group of dCMP then serves as a primer for DNA synthesis along one strand of the viral DNA. (b) After initiation of DNA replication at the ends of the viral DNA, synthesis proceeds by a strand-displacement mechanism (i,ii). The viral E2A region-coded DNA binding protein (DBP) is essential for viral DNA replication and binds to single-stranded viral DNA. A fully displaced single strand (iii) can form a hairpin structure (iv) in which the inverted terminal repeats (ITRs) anneal to form a duplex which is identical to the end of double-stranded viral DNA. The duplex portion of the hairpin structure can serve as a site for another initiation reaction (v) that can then complete the replication cycle to generate two duplex daughter molecules.

Transcription originating from the MLP terminates near the right end of the genome and the late transcripts are processed into a complex array of different messenger ribonucleic acids (mRNAs) which encode most of the structural virion proteins.

Viral DNA replication (**Figure 2**), which begins at about 6–8 h postinfection in permissive cells, requires both virus-coded and cellular factors. The inverted terminal repeats (ITRs) are the only sequences required in *cis* for Ad DNA replication. DNA synthesis is initiated by a protein-priming step involving covalent linkage of deoxycytidine monophosphate (dCMP) to the virus-coded preterminal protein, pTP. The 3′OH of the pTP–dCMP then serves as a primer to initiate DNA synthesis at either end of the genome, which continues through to the 5′ end of the template by a strand displacement mechanism (**Figure 2**) (Lechner and Kelly, 1977). Synthesis is catalysed by the virus-coded DNA polymerase (Pol) and is facilitated by a virus-coded DNA binding protein (DBP). The ITR sequences found at the termini of the displaced parental strand can anneal to form a panhandle structure, which can also function as a template for initiation of DNA synthesis. During or after packaging of the viral DNA into virions the pTP is proteolytically processed to generate terminal protein (TP) which remains covalently attached to the viral DNA and probably serves to protect the DNA ends from cellular nucleases during the next round of infection and replication.

Adenovirus Vectors for Gene Transfer

There are many kinds of adenovirus vectors and many ways of constructing them. At one extreme are nondefective vectors that retain all essential viral genes and have inserts of foreign DNA in nonessential regions of the genome and at the other extreme are vectors from which all viral genes have been deleted and substituted with foreign DNA (up to 36 kb).

First-generation vectors

From the perspective of 'adenovectorology' the most important regions are E1 and E3 (**Figure 1**); the latter is

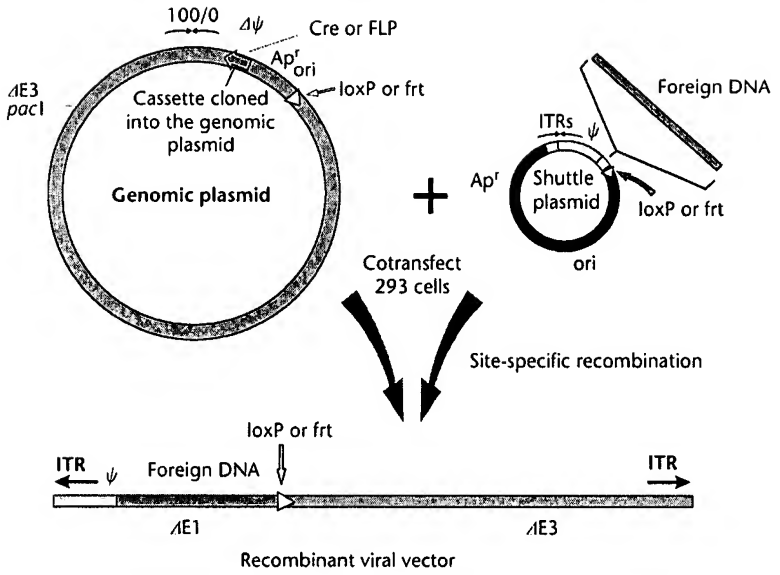

Figure 3 Construction of Ad vectors by site-specific recombination following cotransfection of 293 cells. The method depends on formation of an infectious viral DNA molecule by recombination between two noninfectious plasmids. The genomic plasmid and the shuttle plasmid are able to replicate in cotransfected cells because inverted terminal repeat (ITR) junctions can serve as origins of adenovirus DNA replication (Graham, 1984). However, neither DNA molecule is capable of generating infectious virions – the genomic plasmid because it lacks the packaging signal (ψ) and the shuttle plasmid because it does not encode any viral proteins. High-efficiency site-specific recombination between loxP or frt sites (indicated by an open arrowhead) is catalysed respectively by the bacteriophage P1 recombinase, Cre, or by the yeast 2μ plasmid-encoded recombinase, FLP. In the example illustrated here (Ng and Graham, 2002) the recombinases are expressed from a cassette cloned into the genomic plasmid, but the enzymes can also be expressed from a cassette in the shuttle plasmid or by the cotransfected cells. In any case, the recombinase cassette does not appear in the final vector product.

nonessential and can be deleted without interfering with the ability of the virus to replicate, and the former, though essential, can also be deleted resulting in a defective virus that is propagated in E1-expressing cells such as 293 cells (Graham *et al.*, 1977). The DNA packaging capacity of the virion is limited to approximately 1.8 kb greater than the wild-type genome, and deletion of E1 and E3 sequences can increase the capacity for foreign gene insertion to as much as 8 kb.The most commonly used vectors, generally referred to as first-generation (FG) vectors, have deletions of both E1 and E3 and inserts of foreign DNA in place of E1. Such vectors can efficiently transduce most cells but are defective for replication. They are particularly useful for gene transfer into cultured cells and for gene therapy applications requiring transient gene expression. **See also:** Adenovirus Culture

Strategies for FG vector construction

Numerous techniques have been developed to isolate Ad vectors carrying insertions of foreign DNA but all depend on the fact that the viral DNA is infectious (Graham and van der Eb, 1973) and rely on manipulations of the genome *in vitro* or in bacteria. The viral genome itself can be cloned as an infectious bacterial plasmid (Graham, 1984; Hanahan and Gluzman, 1984), which facilitates many of the manipulations required to construct vectors. One of the simplest and most efficient methods for cloning foreign DNA into the E1 region of Ad vectors is shown in **Figure 3** and is based on recombination between two plasmids, neither of which is infectious, to form an infectious recombinant after cotransfection of 293 cells (Ng and Graham, 2002). Another popular method for constructing vectors utilizes homologous recombination in bacteria to clone foreign DNA into the viral genome. This method has the advantage that in principle, it facilitates insertion in any (nonessential) region of the genome as well as aiding in other kinds of modifications such as introduction of deletions or mutations into the viral DNA (Chartier *et al.*, 1996).

Second-generation vectors

FG vectors are not suitable for long-term expression because they retain most viral genes and can express them at low levels, resulting in an immune response against transduced cells *in vivo*. In attempts to further attenuate the virus, numerous laboratories have created vectors with additional deletions or mutations besides the deletion of E1. These vectors are often referred to as second-generation vectors and may have defects in E4, encoding functions that regulate viral gene expression, or in E2A or E2B, encoding the proteins involved in viral DNA replication. Cell lines complementing the missing viral functions are required for propagation of such vectors. Although the resulting viruses are more severely attenuated than vectors

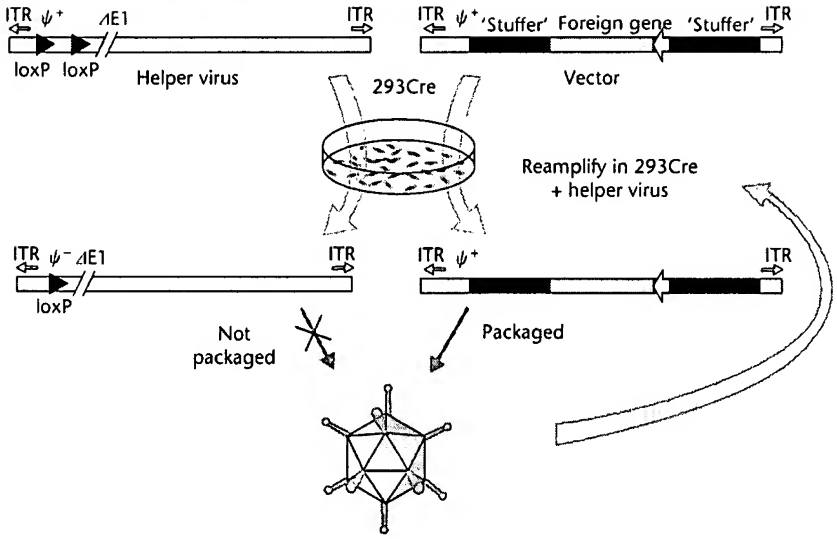

Figure 4 The Cre/loxP system for generating fully deleted (FD) vectors. 293 cells expressing Cre (293Cre) are coinfected with the FD vector and a helper virus bearing a packaging signal flanked by loxP sites. Cre-mediated excision of the packaging signal (ψ) renders the helper virus genome unpackagable, but does not interfere with its ability to provide all of the necessary *trans*-acting factors for propagation of the FD vector. The titer of the FD vector is increased by serial passage in helper virus-infected 293Cre cells. The FD vector need contain only those Ad *cis*-acting elements required for DNA replication (inverted terminal repeats (ITRs)) and encapsidation (ψ); the remainder of the genome consists of the desired transgene and non-Ad 'stuffer' sequences.

with just E1 deleted, it is not clear that these additional modifications to the virus have eliminated the inflammatory response induced by administration of high doses of vector. Indeed, it was the high dose of a second-generation vector with deletions in E1 and E2A that was used in a clinical trial in 1999 that resulted in the death of one patient as a result of acute respiratory distress syndrome and multiple-organ failure (Beardsley, 2000).

High-capacity systems

The only sequences needed in *cis* for viral DNA replication and packaging of DNA into virions are ITRs of approximately 100 base pairs (bp) and a packaging signal (ψ) located at the left end of the genome and spanning approximately 200 bp. Thus, if all necessary gene products are provided in *trans*, then virtually the entire genome can be deleted and substituted with as much as 36 kb of foreign DNA. This is the basis for development of fully deleted (FD) vectors, which are currently the best available Ad vectors for long-term expression *in vivo*. Ideally, one would like to be able to propagate FD vectors in 'helper' cells that express all the functions needed in *trans* for virus replication, but no such cell lines are currently available. Consequently, FD vectors must be propagated in the presence of a helper virus that provides all the viral functions and virion capsid proteins needed for virus replication. Thus, FD vectors are often referred to as 'helper-dependent' vectors. Although the earliest Ad vectors (generated before complementing 293 cells became available) were helper dependent, these were inconvenient to work with because the helper viruses often overgrew the mixed population and represented a major fraction of the final vector preparations. A workable solution to this quandary was developed by using a site-specific recombination system to excise the packaging signal from the helper virus. This prevents packaging of the helper virus genome into virions during coreplication of helper and vector in 293 cells that express the recombinase (Parks *et al.*, 1996; Ng *et al.*, 2002). The system is illustrated in **Figure 4**.

Longevity and Magnitude of Gene Expression

With the development of a practical system for producing large amounts of pure FD vectors, it was possible to carry out studies in animals to compare the toxicity and duration of transgene expression from FG versus FD vectors. In both mice and larger animals, such as baboons, FG vectors administered intravenously, resulting in delivery of the virus mainly to the liver, caused significant liver toxicity with elevated serum levels of liver enzymes, hepatocellular degeneration and necrosis and inflammation. Acute toxicity was less and chronic effects were absent or minimal following administration of FD vectors. In addition, expression of a reporter gene cloned in an FD vector persisted for nearly two years in baboons whereas expression from an FG vector lasted only 1–2 weeks (Morral *et al.*, 1999; Muruve *et al.*, 2004).

Safety Features

Potential pathogenesis

Ads cause a variety of diseases in their permissive hosts, including respiratory infections, keratoconjunctivitis and enteric infections. In some species they can cause hepatitis. Vaccines against a few human adenovirus serotypes are available and until recently were used by the United States military to vaccinate recruits against acute respiratory disease caused by serotypes 4 and 7, but adenovirus vaccines are not used for the general population. This is probably due in part to the fact that, except in immune compromised individuals, adenovirus infections are not usually life-threatening and are self-limiting and in part to the large number of different serotypes that infect humans.

Attenuation of vectors

FG vectors that have a deletion of E1 are strongly attenuated and unable to replicate following low multiplicity infection of most normal cells because E1 functions are required for efficient expression of other viral genes. However, $E1^-$ viruses can exhibit 'leaky' expression of viral genes in some cells, particularly at high multiplicities, and very high concentrations of just the virions themselves may be toxic to cells and tissues and may cause an inflammatory response. It would be essentially impossible for FG vectors to spread to uninfected cohorts from a treated patient because of the negligible levels of *de novo* virus production. However, production of FG vectors in 293 cells, the most commonly used cell line can result in vector preparations with contaminating $E1^+$ replication-competent adenovirus (RCA). This occurs by homologous recombination with Ad5 sequences in 293 cells and results in a virus that has lost the foreign DNA insert in E1 and regained a wild-type E1 region. The consequences of administration of mixtures of FG vectors and RCA viruses are unpredictable since, if the latter were present at high concentrations and the virus preparations were used at high concentrations, RCA could result in complementation of the E1 defect of the vectors and coreplication in coinfected cells resulting in amplification of the vector. As a result, considerable effort has been devoted to minimizing RCA contamination in clinical preparations of Ad vectors and new E1-complementing cell lines have been developed that reduce or eliminate the chances of RCA formation. FD vectors are likely to be the safest Ad-based gene delivery system and could be widely used if efficient methods can be developed for their production and purification in large amounts.

Applications

FG vectors are easy to engineer, propagate and purify and have numerous uses whenever efficient gene delivery and high-level expression are desired. They are thus excellent research tools and will be used increasingly as novel genes are discovered and their products become subjects for investigation. Because the vectors can deliver genes encoding antigens and express them at high levels *in vivo* in any mammalian species, they are excellent candidates as recombinant viral vaccines. Vectors capable of immunizing animals against rabies, herpesviruses, rotaviruses and coronaviruses have all been developed. Among the most important applications, for which FG vectors are particularly suited, is immunotherapy of cancer. Here, transient expression would be preferred over long-term expression, and the inflammatory response associated with administration of FG vectors may be advantageous. Several FG vectors have been produced that express a variety of cytokines and other immunomodulatory proteins (Addison *et al.*, 1995; Putzer *et al.*, 1997). These have been tested in animal tumour models with encouraging results and some have been used in clinical trials (Stewart *et al.*, 1999).

Other applications in cancer therapy make use of FG vectors that are able to express cytotoxic products or that exploit the ability of Ads to replicate in and lyse cells by developing mutant $E1^+$ viruses designed to replicate preferentially in tumour cells ('oncolytic' Ads). Ad vectors encoding the tumour suppressor gene p53 have shown some efficacy when combined with chemotherapy in the treatment of head and neck cancer and non-small cell lung carcinoma. Based on these results, in 2003 China became the first country in the world to approve a gene therapy vector (the Adp53 vector Gendicine) for the treatment of human disease. More recently an oncolytic Ad (the E1b-mutant H101) was also approved for sales in China. A promising approach for cancer therapy is to combine immunomodulatory genes with an oncolytic virus backbone to generate an 'armed' oncolytic virus.

The high efficiency of Ad gene transfer to many cell types is an advantage for many applications, but it could be a major drawback to *in vivo* gene therapy if the therapeutic gene is toxic when expressed in tissues that are not the primary target of the treatment. Confining the activity of the therapeutic gene to the target tissue should rescue normal or untargeted tissue from inadvertent transduction and subsequent destruction. One way to limit transgene activity is to position regulatory elements from tissue- or tumour-specific promoters such that they control transcription of the therapeutic gene in the vector (reviewed by Sadeghi and Hitt, 2005). Many transcriptionally targeted vectors have been developed and some have been or are being tested in clinical trials for cancer, including oncolytic viruses in which E1a gene expression is controlled by prostate-specific promoters. As an alternative, strategies are being developed to redirect binding of Ad vectors to receptors highly expressed on target cells. This is particularly important for cancer therapies because many different types of tumour cells have reduced levels of the wild-type virus receptor on the surface. Although this approach has

been quite challenging so far, effectively redirecting Ad vectors could have an enormous impact on the field of cancer gene therapy because the increased specificity would allow vectors to carry much more effective or toxic payloads without an increased risk of damage to normal tissue. **See also**: Delivery Targeting in Gene Therapy; Expression Targeting in Gene Therapy

Although FD vectors are technically more difficult to engineer, propagate and purify than FG vectors, they have a much higher therapeutic index (the benefit/risk ratio) and give much longer expression *in vivo*. FD vectors may therefore find use in 'classical' gene therapy such as enzyme replacement where the desired outcome is permanent expression of the transgene product.

In summary, Ad vectors come in many forms and have great versatility and high efficacy when designed and used appropriately. They will play an increasingly important role as agents for gene transfer into mammalian cells.

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